used the data from a total of 500 advanced cancer patients, who received personalized peptide vaccination conducted between October 2000 and October 2008, to investigate biomarkers that are predictive of their overall survival. Furthermore, we used samples from long-term survivors (more than 900 days of overall survival) and short-term survivors (less than 300 days of overall survival) with advanced castration-resistant prostate cancer (CRPC) under treatment with personalized peptide vaccination. It is well known that advanced CRPC patients rarely survive more than 2 years even if they receive global standard chemotherapy combined with hormone therapy.[12] Therefore, although only 43 patients were examined in subgroup analysis, the clinical benefits in the long-term survivors should be sufficiently large for the statistical analysis to identify definite biomarkers easily if any.

Results

Patient characteristics, immunological and clinical responses. The demographic, immunological responses and clinical characteristics of the 500 patients with advanced cancer are listed in **Table 1A and B**. The most frequent symptom of toxicity in the personalized peptide vaccination was a local skin reaction at injection sites. These symptoms were manageable through routine interventions as reported previously.[13-29] The best response to the personalized peptide vaccination was assessed in 436 patients. No complete responses (CR) were observed in either group. Forty-three patients (10%) had partial response (PR) and 144 patients (33%) had stable disease (SD). The remaining 249 patients (57%) had progressive disease (PD) without responses. Most of these clinical responses were already reported.[13-29] The response rate and disease control rate during the personalized peptide vaccination were 9.9 and 42.9%, respectively.

Correlation between overall survival and immune responses. The median follow-up for all 500 patients was 9.1 months (range, 1–105 months). Forty-five patients (9%) were alive at the end of the study (October 2009). Four hundred and forty-five patients died from advanced cancer and 10 patients died of other causes. The median overall survival time was 9.9 months with 1- and 3-year survival rates of 43 and 10.7%, respectively (**Fig. 1A**). Peptide-specific cellular and humoral immune activities were measured at 6-week intervals as long as patient samples were available. The total numbers of evaluable patients for CTL and IgG responses during the personalized peptide vaccination were 332 and 300, and positive results in CTL and IgG responses after the sixth vaccination were detected in 199 (60%) patients and in 187 (62%) patients, respectively. The median overall survival for patients with a positive IgG response was significantly longer than that for patients with a negative IgG response ($p = 0.0015$ by log-rank test; **Fig. 1C**), while an association between CTL response status and overall survival was not observed ($p = 0.167$ by log-rank test; **Fig. 1B**).

Analysis of predictors of overall survival. Cox proportional hazards regression analysis was performed to determine factors that are predictive of overall survival in the 500 patients listed above (**Table 2**). In univariate regression analysis, performance status ($p < 0.0001$), counts of lymphocytes ($p < 0.0001$), IgG response and age ($p = 0.002$) were found to be associated with survival. Gender, CTL response, HLA typing and vaccine interval were not significant factors. Forward stepwise multivariate analysis showed that only performance status ($p < 0.0001$; hazard risk 2.295; 95% CI, 1.653–3.188), counts of lymphocytes ($p = 0.0095$; hazard risk 1.472; 95% CI, 1.099–1.972) and IgG response ($p = 0.0116$; hazard risk 1.455; 95% CI, 1.087–1.948) were independent predictors of overall survival. None of the other variables were significant predictors of overall survival.

Comparison of immune responses between short- and long-term survivors. To statistically confirm the superiority of IgG response as a predictor to CTL response, samples from 20 patients who survived more than 900 days (long-term survivors) and those from 23 patients who died within 300 days (short-term survivors), among 174 patients with CRPC who received personalized peptide vaccination, were analyzed further. There were no statistical differences between the two groups with regard to clinical and pathological characteristics at the time of entry (**Table 3**). The only apparent difference was overall survival after the vaccination. Median survival times of long- and short-term survivors used for the analysis were 1,483 days and 189 days, respectively.

The frequencies of selection of each peptide candidate at the first vaccination between long- and short-term survivors were investigated to address if the peptides used were different between the two groups. There were no significant differences in the frequencies of selection of each peptide at the first vaccination between the two groups.

The levels of IgG reactive to each of the vaccinated peptides were measured for 21 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods, and the representative results were given in **Table 4A and B**. The post-vaccination samples were not available from two short-term survivors. In short-term survivors, the numbers of peptides, against which a more than two-fold increase in IgG was observed, were 0 peptide in 10 patients, 1 peptide in 7 patients, 2 peptides in 3 patients and 3 peptides in 1 patient. In long-term survivors, numbers of peptides, to which increased IgG responses were observed, were 0 peptide in 3 patients, 1 peptide in 3 patients, 2 peptides in 5 patients, 3 peptides in 6 patients and 4 peptides in 3 patients ($p = 0.000282$). To better represent n-fold increase in IgG levels, the results were drawn in **Figure 2**, in which the vertical bars denote log 10 scores. In short-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 16 patients, 1 peptide in 2 patients, 2 peptides in 2 patients and 3 peptides in 1 patient. In long-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 5 patients, 1 peptide in 6 patients, 2 peptides in 5 patients and 3 peptides in 4 patients ($p = 0.00045$).

CTL activity against each of the vaccinated peptides was measured in 17 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods (**Table 4A and B**). The post-vaccination peripheral blood mononuclear cells (PBMCs) needed for measurement of CTL responses were not available from 9 short-term survivors primarily because of rapid progression of cancer. In short-term survivors, the numbers

Table 1A. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Total		Groups of cancer											
			Prostatic cancer		Colorectal cancer		Pancreatic cancer		Gastric cancer		Brain tumor		Cervical cancer	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	500		174	35	74	15	50	10	42	8	33	7	28	6
Average Age, years	61.8		67.9		58.5		64.8		58.7		49.6		49.9	
Standard deviation	12.8		7.8		12.3		8.8		12.3		20.3		12.4	
Sex														
Male	353	71	174	100	52	70	32	64	29	69	18	55	-	
Female	147	29	-		22	30	18	36	13	31	15	45	28	100
Performance status (ECOG)														
0	333	67	144	83	47	64	33	66	20	48	7	21	16	57
1	118	24	25	14.5	23	31	16	32	16	38	6	19	9	32
2	31	6	1	0.5	4	5	0	0	6	14	8	24	3	11
3	18	3	4	2	0	0	1	2	0	0	12	36	0	0
Peptides bind for HLA														
A2	139	28	48	28	16	22	15	30	14	33	8	24	11	39
A24	332	66	109	63	58	78	31	62	28	67	25	76	17	61
A3-supretype	6	1	4	2	0	0	0	0	0	0	0	0	0	0
Mixed type	23	5	13	7	0	0	4	8	0	0	0	0	0	0
Average times of vaccination	14.7		17		13.9		16.3		9.8		13.1		14	
Standard deviation	15		18.9		11.8		14.3		9.8		11.2		9.9	
Treatment														
Vaccination alone	331	66	109	63	47	64	11	22	34	81	14	42	28	100
Combination	169	34	65	37	27	36	39	78	8	19	19	58	0	0
CTL response														
No. of evaluable case	332		111		60		40		25		26		20	
yes	199	60	75	68	32	53	26	65	15	60	17	65	13	65
no	133	40	36	32	28	47	14	35	10	40	9	35	7	35
IgG response														
No. of evaluable case	300		105		48		41		21		22		12	
yes	187	62	77	73	27	56	21	51	14	67	11	50	7	58
no	113	38	28	27	21	44	20	49	7	33	11	50	5	42
Best clinical response														
No. of evaluable case	436		155		68		41		35		30		23	
PR	43	10	29	19	1	1	4	10	0	0	5	16	3	13
SD	144	33	36	23	23	34	23	56	8	23	11	37	7	30
PD	249	57	90	58	44	65	14	34	27	77	14	47	13	57
Response rate (%)	9.9		18.7		1.5		9.8		-		16.7		13	
Disease control rate (%)	42.9		41.9		35.3		65.9		22.9		53.3		43.5	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

of peptides, against which increased CTL responses were observed, were 0 peptide in 4 patients, 1 peptide in 6 patients and 2 peptides in 4 patients. In long-term survivors, the numbers of peptides, against which increased CTL responses were observed, were 0 peptide in 5 patients, 1 peptide in 12 patients, 2 peptides in 1 patient and 3 peptides in 2 patients ($p = 0.827009$).

Discussion

This study showed that both lymphocyte counts prior to the vaccination and increased IgG response to the vaccinated peptides, along with performance status, well correlated with overall survival of advanced cancer patients who received personalized peptide vaccination. Lymphocyte counts prior to vaccination shall be a biomarker primarily because lymphocytes are absolutely required for vaccine-mediated immune boosting. In addition, lymphopenia is recently reported to be an independent prognostic factor for overall survival in advanced cancers.[34] In contrast to lymphocyte counts, one might question why IgG response, but not CTL response, is a biomarker of the effectiveness of the peptide vaccination given that the vaccination primarily activates peptide-specific CTLs, but not B cells. We also brought up the same question when reporting on IgG responses as a biomarker following an investigation of 211 patients under treatment with personalized peptide vaccination.[10] Therefore, we extended that study in the present work and report convincing results showing that IgG response is superior to

Table 1B. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Groups of cancer											
	NSCLC		RCC		Melanoma		Brest cancer		Urotherial cancer		Others	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	22	4	13	3	12	2	11	2	10	2	31	6
Average Age, years	60.5		57.8		57.3		54.3		66.6		63.6	
Standard deviation	12.4		11.2		18.2		11.4		10.7		11.9	
Sex												
Male	11	50	11	85	7	58	0	0	9	90	16	52
Female	11	50	2	15	5	42	11	100	1	10	15	48
Performance status (ECOG)												
0	14	64	10	77	7	58	5	46	6	60	16	52
1	5	23	2	15	3	25	4	36	3	30	8	26
2	3	13	1	8	2	17	1	9	1	10	7	22
3	0	0	0	0			1	9	0	0	0	0
Peptides bind for HLA												
A2	4	18	3	23	4	33	4	36	4	40	8	26
A24	18	82	9	69	8	67	7	64	6	60	16	52
A3-supretype	0	0	0	0	0	0	0	0	0	0	2	6
Mixed type	0	0	1	8	0	0	0	0	0	0	5	16
Average times of vaccination	13.8		23.5		12.3		9		11.9		10.8	
Standard deviation	15.4		15		6.6		9.8		6		13.4	
Treatment												
Vaccination alone	22	100	12	92	12	100	4	36	9	90	29	94
Combination	0	0	1	8	0	0	7	64	1	10	2	6
CTL response												
No. of evaluable case	11		10		8		6		3		12	
yes	6	55	2	20	6	75	1	17	2	67	4	33
no	5	45	8	80	2	25	5	83	1	33	8	67
IgG response												
No. of evaluable case	12		9		7		4		3		16	
yes	7	58	5	56	5	71	4	100	2	67	7	44
no	5	42	4	44	2	29	0	0	1	33	9	56
Best clinical response												
No. of evaluable case	21		12		11		10		7		23	
PR	0	0	0	0	0	0	0	0	1	14	0	0
SD	11	52	9	75	5	45	1	10	2	29	8	35
PD	10	48	3	25	6	55	9	90	4	57	15	65
Response rate (%)	-		-		-		-		14.3		-	
Disease control rate (%)	52.4		75		45.5		10		42.9		34.8	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

CTL response in predicting the overall survival of advanced cancer patients under treatment with personalized peptide vaccination.

It is obvious that cellular immune responses shall be an important marker if appropriate assay conditions are defined and used. However, the current available T cell assays possess insufficient sensitivity and reproducibility for monitoring immune responses in vaccinated patients. Various T cell assays for quantifying and characterizing antigen-specific T cell responses, including ELISPOT, ELISA, intracellular cytokine staining (ICS), ^{51}Cr-release cytotoxicity assay, peptide-MHC multimer and proliferation assay (^{3}H-thymidine uptake and CFSE), have been extensively studied.[4,30,31] Using these T cell assays, increasing numbers of studies have reported significant correlations between clinical benefits and immunological responses in a limited number of patients.[4,30,31] However they are often inconsistent and unreproducible in other studies, because no universal standards have been established in the current T cell assays, which continue to be modified on a regular basis.[4,30,31] In fact, we have already tried several T cell assays, including delayed type hypersensitivity test and cytotoxicity assay, in our vaccinated patients, but their results were no better than the CTL precursor assay that we employed in the current study.[10] We also employed ELISPOT assay with the similar results (Noguchi M, et al., unpublished results). Therefore, we think that optimization and

standardization of T cell assay protocols, including the analysis, interpretation and reporting of data, may be crucial for future development of immune monitoring in cancer patients.[4,30,31] Nevertheless, it should be also noted that T cell assays have their inherent limitations. Even if innovated technologies are introduced and assay protocols are sophisticated, it will be difficult to dramatically improve their performance characteristics, such as sensitivity and reproducibility, because the frequencies of antigen-specific T cells are usually quite low even after vaccination.[5,6]

One might have several questions with regard to relationship between peptide-specific CTL responses and peptide-specific IgG responses, but we found no statistically significant correlation between the increased IgG responses and the increased CTL responses in 300 patients shown in **Table 1A and B** as well as 43 patients shown in **Table 4A and B**. We previously reported that both IgG and CTL responses were augmented in the samples after 6th vaccination from the majority of patients who showed PR responses.[19,23,25] We also demonstrated that there were no significant differences in overall survival between patients showing both CTL and IgG responses and those showing only IgG response.[10,11] These results suggest that boosted CTL responses are involved in tumor reduction, but not necessarily involved in prolonged overall survival.

We investigated the correlation between pre-vaccination lymphocyte counts and the induction of IgG responses in the 43 patients listed in **Table 4A and B**. As a result, there was no significant correlation between them. In addition, we addressed if boosted IgG responses to the vaccinated peptides were associated with concomitant increase of peptide-specific IgG to non-vaccinated peptides in the patients showing longer survivals shown in **Table 4A and B**. As a result, no such concomitant increase was observed in the majority of long survivors as well as short survivors listed in **Table 4A and B**. These results suggest that the boosting effect was really limited to the vaccinated peptides.

There could be several possible explanations for these unexpected results. Firstly, to the best of our knowledge, none of the previously reported studies involved more than several hundred cases under a single concept (personalization of peptide selection) of therapeutic peptide vaccination for advanced cancer patients. Although some of the clinical trials of peptide vaccination identified CTL response as a biomarker that predicts overall survival,[2,4,10,30] the numbers of patients were too small to obtain significant results. Furthermore, the clinical benefits of those peptide vaccination trials were not sufficiently large to enter randomized phase III trials. A number of poorly validated or controversial markers made it difficult to obtain approval of cancer vaccines as drugs. Indeed, there are no prospectively defined markers validated in large phase II or III studies at the time of writing.[7-9] Therefore, IgG response, but not CTL response, to the vaccinated peptides or proteins has the possibility to become a true biomarker that is predictive of the overall survival of cancer patients under treatment with cancer vaccine. In line with our observations, other researchers have also recognized the significance of B cell responses induced by vaccination with tumor antigens. Secondly, we previously reported that the personalized peptide vaccination mainly induced infiltration of CD45RO$^+$ T cells, but not that of CD8$^+$ T cells or CD20$^+$ B cells.[31] The results suggest that personalized peptide vaccination initially induced CD45RO$^+$ memory helper T cells to infiltrate into tumor sites, which in turn facilitated the proliferation of CD8$^+$ CTLs and B cells. Consequently, the activated CTLs eliminated cancer cells, while the activated B cells differentiated into plasma cells, which in turn produced IgG specific to the vaccinated peptides. Although the precise mechanisms, in which helper CD4$^+$ T cells are activated after vaccination with HLA class I-restricted

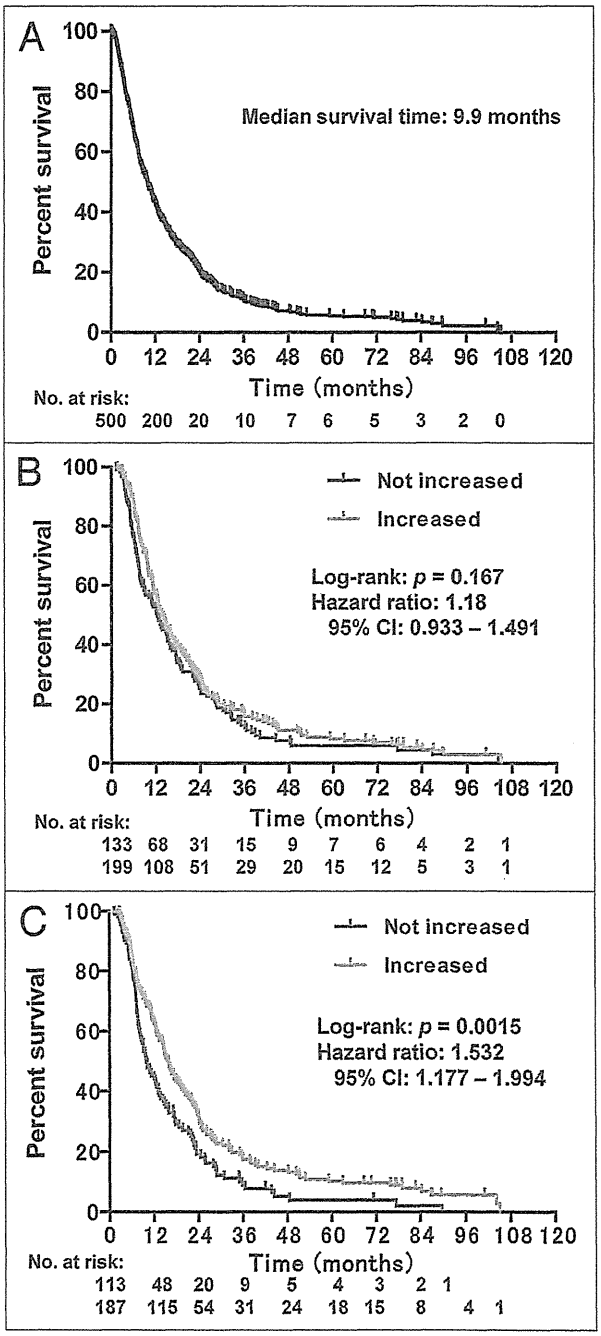

Figure 1. (A) Overall survival in 500 patients with advanced cancer treated by personalized peptide vaccination. Overall survival curves according to peptide-specific cellular (B) and humoral (C) immune response status.

Table 2. Univariate and multivariate analysis for overall survival using Cox regression models

	Univariate			Multivariate		
Factor	**p**	**HR**	**95% CI**	**p**	**HR**	**95% CI**
Performance status (ECOG) ≥ v < 1	<0.0001	2.4560	1.990–3.030	<0.0001	2.2950	1.653–3.188
Counts of lymphocytes < v ≥ 1,500/μL	<0.0001	1.6810	1.362–2.074	0.0095	1.4720	1.099–1.972
IgG responses no v yes	0.0015	1.4970	1.167–1.919	0.0116	1.4550	1.087–1.948
Age < v ≥ 63	0.0020	1.3420	1.113–1.617	-	-	-
Gender Male v Female	0.0984	0.8420	0.686–1.033	-	-	-
CTL responses no v yes	0.1587	1.1800	0.937–1.486	-	-	-
HLA typing A24 v others	0.2504	0.8900	0.729–1.086	-	-	-
Vaccine interval 1 week v ≥ 2 weeks	0.2117	0.8760	0.712–1.078	-	-	-

Lymphocyte and patient age are based on median values, and the remaining are treated as dichotomous variables.

Table 3. Baceline patient characteristics

	Long survivors		Short survivors		
	No	**%**	**No**	**%**	**p**
No. of patients	20		23		
Age, years					
Median	71		64		0.152
Range	54–78		50–80		
ECOG performance status					
0	20	100	20	87	0.236
1			3	13	
HLA typing					
A24	10	50	13	57	0.761
A2	8	40	8	35	
A24 and A2	2	10	2	8	
PSA, ng/ml					
Median	34.5		83		0.404
Range	2–330		2–296		
Gleason score					
7	6	30	3	13	0.299
8	9	45	10	43.5	
9	5	25	10	43.5	
Site of metastasis					
No	3	15	2	9	0.651
Bone only	14	70	17	74	
Bone and node	2	10	3	13	
Node/organ	1	5	1	4	
Progression free survival time, days					
Median	57		43		0.042
Range	14–926		14–96		
Survival time, days					
Median	1483		189		<0.0001
Range	699–2811		79–297		

peptides, still remain to be clarified, one possibility is that the peptides employed in this study may be presented not only in HLA class I but also in HLA class II and recognized by both CD8 and CD4 T cells, as has been reported in the PSA peptide at position 248–257 in prostate patients by our group and also in the Melan A 26–35 (A27L) peptide in melanoma patients.[32,33] Alternatively, the peptides employed in this study may be recognized by $CD4^+$ T cells on HLA class I molecules without requirement of CD8

Table 4A. Comparison of immune responses between short- and long-term survivors

Short-term survivors (n = 23)							
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
1	SART3-109	0	NT	NA	492	1221	≥2
	Lck-208	0	NT	NA	11	18	negative
	Lck-488	0	NT	NA	15	20	negative
	SART3-315	0	NT	NA	30	27	negative
2	SART3-109	53	183	≥2	456	3123	≥2
	Lck-488	159	0	negative	320	310	negative
	ART1-170	1312	0	negative	<10	<10	negative
	SART3-315	77	189	≥2	<10	<10	negative
3	SART2-161	899	0	negative	36	38	negative
	Lck-208	323	108	negative	<10	<10	negative
	Lck-486	101	0	negative	118	144	negative
	SART3-315	53	69	negative	35	30	negative
4	SART3-109	41	NT	NA	22	14	negative
	Lck-208	67	NT	NA	<10	<10	negative
	Lck-486	78	NT	NA	107	92	negative
	ART4-75	79	NT	NA	NT	NT	NA
5	CypB-172	212	NT	NA	<10	1211	≥10
	HNRL-501	477	NT	NA	<10	18	≥10
	ppMAPkkk-294	0	NT	NA	12	13	negative
6	PAP-213	159	0	negative	34	39	negative
	PSA-248	55	0	negative	273	2138	≥2
	SART3-315	449	0	negative	<10	<10	negative
	PSA-152	516	61	negative	<10	<10	negative
7	UBE-43	0	NT	NA	308	NT	NA
	UBE-208	223	NT	NA	73	NT	NA
	PSCA-21	0	NT	NA	143	NT	NA
	EGFR-479	74	NT	NA	68	NT	NA
8	UBE-43	0	NT	NA	544	NT	NA
	PSCA-21	56	NT	NA	358	NT	NA
	PTHrP-42	0	NT	NA	176	NT	NA
	Her2/neu-484	0	NT	NA	227	NT	NA
9	SART3-302	608	NT	NA	229	19363	≥10
	Lck-422	0	NT	NA	14	215	≥2
	WHSC2-103	0	NT	NA	48	70	negative
	UBE2V-43	0	NT	NA	35	59	negative
10	SART3-109	5561	0	negative	274	283	negative
	Lck-488	0	0	negative	98	96	negative
	MRP3-1293	0	0	negative	78	76	negative
	PAP-213	0	0	negative	68	69	negative

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

11	SART3-309	NT	NT	NA	199	537	≥2
	CypB-129	NT	NT	NA	804	530	negative
	UBE-43	NT	NT	NA	41	28	negative
	HNRL-501	NT	NT	NA	35	26	negative
12	SART2-93	0	0	negative	12	12	negative
	Lck-208	68	0	negative	15	11	negative
	Lck-486	123	348	≥2	21	<10	negative
	CypB-91	0	0	negative	15	11	negative
13	CypB-172	488	1000	≥2	151	<10	negative
	Lck-422	0	0	negative	12	<10	negative
	MAP-294	0	0	negative	41	21	negative
	HNRL-501	0	0	negative	15	16	negative
14	SART3-109	0	2045	≥10	9524	7283	negative
	Lck-208	0	2246	≥10	0	0	negative
	Lck-488	118	184	negative	70	86	negative
	PSA-248	0	0	negative	8	11	negative
15	SART3-109	0	0	negative	561	780	negative
	PAP-213	0	0	negative	112	125	negative
	PSA-248	0	0	negative	251	271	negative
	PSA-152	109	0	negative	29	<10	negative
16	SART3-302	0	931	≥10	251	223	negative
	CypB-172	0	0	negative	312	350	negative
	Lck-246	0	4326	≥10	186	199	negative
	ppMAPkkk-294	0	0	negative	132	126	negative
17	Her2/neu-553	0	NT	NA	31	NT	NA
	EZH2-291	0	NT	NA	26	NT	NA
	PTHrP-102	0	NT	NA	10	15	negative
	PSA-248	0	NT	NA	45	822	≥10
18	PAP-213	0	1289	≥10	534	18980	≥10
	PSA-248	0	0	negative	103	9855	≥10
	Her2/neu553	0	302	≥10	59	89	negative
19	SART3-109	0	0	negative	879	930	negative
	Lck-488	0	0	negative	641	663	negative
	PAP-213	0	191	≥10	143	138	negative
20	SART3-302	0	0	negative	11	10	negative
	UBE2V-43	0	0	negative	40	43	negative
	HNRL-501	0	0	negative	10	12	negative
	EZH2-569	0	130	≥10	38	39	negative
21	SART3-109	0	0	negative	287	7040	≥10
	Lck-486	0	0	negative	232	334	negative
	PAP-213	0	131	≥10	91	21230	≥10
	EZH2-291	753	0	negative	341	14258	≥10

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

22	SART2-161	0	4923	≥10	24	90	≥2
	SART3-109	318	0	negative	141	151	negative
	Lck-486	0	0	negative	39	41	negative
	MRP3-1293	262	0	negative	30	30	negative
23	SART3-109	0	NT	NA	945	7675	≥2
	Lck-486	0	NT	NA	18	18	negative
	MRP3-1293	0	NT	NA	16	16	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

molecules, as has been reported on T cell receptor-engineered $CD4^+$ T cells.[34] Although we have no data on the association between HLA class II types in the vaccinated patients and anti-peptide IgG responses in the current study, this important issue will be addressed in further studies. Biological roles of peptide-specific IgG also need to be elucidated in the near future.

Increases IgG responses to the vaccinated peptides in patients showing longer survival could be, at least in part, in reflection of their better immune-competence with regard to helper T cell functions and subsequent B cell responses, although biomarkers predictable of better immune-competence with regard to favorite clinical benefits in response to peptide vaccinations are presently unclear. This issue is now under investigation and our preliminary results suggest that serum levels of C-reactive protein could be one of them (Noguchi M, et al. unpublished results). At the literature level, a number of prognostic factors have been evaluated with respect to their roles in determining the treatment strategy and ability to predict the response to therapy. Recent reports have shown some significant prognostic factors for CRPC patients. Smaletz et al. reported that performance status, lactate dehydrogenase (LDH), PSA and alkaline phosphate were significant prognostic factors of overall survival in HRPC patients.[35] Halabi et al. reported that performance status, Gleason sum, LDH, alkaline phosphatase, PSA, hemoglobin and visceral metastases were associated with survival in CRPC patients.[36] Unlike these reports, we identified the number of lymphocytes before vaccination and IgG responses after vaccination. These factors were not included in the other reports because most patients in the above studies were treated without specific active immunotherapy.

To address whether or not the long-term survived HRPC patients shown in **Table 4A and B** were different from "better performing, more likely to survive" patients who are not treated with cancer vaccines, we compared the results shown in this study with those of the TAX327 study of docetaxel-based regimens without the vaccine treatment, as a well known historical control, primarily because the disease conditions of HRPC patients in the TAX327 study were similar to those of this study subjects.[13,37] Namely, in the TAX327 study, a randomized, nonblinded, multinational phase III study involving 1,006 men with HRPC, they had a median survival of 16 to 20 months.[13,37] In that study, there were 800 deaths (80%) of 1,006 patients within 18 months of follow-up.[38] Therefore, long-term survivors for more than 30 months (900 days) shown in **Table 4A and B** could be considered to benefit from the peptide vaccination, and thus could be different from better performing HRPC patients who received the standard therapy without cancer vaccines. Of note, the beneficial roles of our personalized peptide vaccination have been also clearly demonstrated in the recently conducted randomized trial in consideration of the pre-existing host immunity.[39] Although several papers[2,4,39] have been reported on the relationships between lymphocyte counts and survival in advanced cancers, there have been no publications regarding antibody responses after peptide vaccinations and survival in cancer patients. Because all of our data were derived from the cancer patients that might have received a survival benefit from vaccinations, we cannot know whether the patients who were able to mount an antibody response and who were not lymphopenic were in fact more likely to control the cancer (and survive longer) even if they did not receive the vaccine. To address this issue, we will need to examine anti-peptide IgG responses after vaccinations with antigen peptides that do not affect patient survival. However, it would be very difficult for us to obtain such data.

One might have a question whether the IgG responses to the vaccinated peptides are unique to the peptides used in this study or widely observed in peptide vaccines conducted in other groups. Unfortunately, to our knowledge, no other groups have examined anti-peptide IgG responses after peptide vaccinations in the literature. Therefore, it would be impossible for us to decide whether the IgG responses that we detected in this study are unique to our peptide vaccines or not. Also, we do not know at the present time whether anti-peptide IgG responses are useful in general as an indicator of survival in cancer patients without vaccinations, because all of our data were derived from the cancer patients that received peptide vaccinations. Of note, however, the methods to identify the peptides used in this study are largely different from those by other groups. We at first established tumor-specific CTL clones and lines in culture of patients' PBMCs and autologous tumor cell lines, followed by identification of genes

Table 4B. Comparison of immune responses between short- and long-term survivors

		Long-term survivors (n = 20)					
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
24	SART2-93	83	0	negative	23	106	≥2
	SART3-109	0	922	≥10	252	11618	≥10
	Lck-488	116	85	negative	120	337	≥2
	PSMA-624	154	0	negative	58	276	≥2
25	Lck-208	71	0	negative	<10	<10	negative
	ART1-170	101	74	negative	<10	35	≥10
	ART4-75	101	0	negative	24	510	≥10
	CypB-84	141	0	negative	<10	<10	negative
26	CypB-129	0	0	negative	43	149	≥2
	Lck-246	0	0	negative	1155	22853	≥10
	HNRL-501	120	729	≥2	<10	51	≥10
	EIF-51	169	0	negative	NT	NT	NA
27	SART3-109	0	0	negative	1107	26809	≥10
	SART3-315	0	0	negative	<10	151	≥10
	Lck-208	60	0	negative	169	142	negative
	PSA-152	106	0	negative	81	114	negative
28	SART3-109	0	700	≥10	57	67	negative
	Lck-488	194	0	negative	90	94	negative
	MRP3-1293	108	0	negative	20	37	negative
	PSMA-624	133	0	negative	55	66	negative
29	SART2-161	143	0	negative	27	15	negative
	SART3-109	0	1032	≥10	263	418	negative
	Lck-488	208	0	negative	81	56	negative
	PSA-248	0	0	negative	70	78	negative
30	UBE2V-43	0	720	≥10	48	5083	≥10
	EIF4EBP-51	261	294	negative	23	83	≥2
	PSA-170	565	0	negative	62	32	negative
	EGF-R-479	0	0	negative	71	67	negative
31	SART3-109	80	3502	≥10	121	115	negative
	Lck-486	0	0	negative	45	35	negative
	SART1-690	0	0	negative	301	426	negative
	SART2-899	677	147	negative	98	260	≥2
32	MAP-432	64	480	≥2	178	1259	≥2
	Lck-246	0	720	≥10	11	451	≥10
	Lck-422	0	130	≥10	13	15	negative
	UBE-43	0	0	negative	15	1534	≥10
33	SART3-309	59	0	negative	142	179	negative
	CypB-172	324	0	negative	129	121	negative
	WHSC-103	70	0	negative	<10	<10	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4B. Comparison of immune responses between short- and long-term survivors (continued)

	WHSC-141	0	0	negative	<10	<10	negative
34	SART3-315	0	0	negative	NT	NT	NA
	PSA-248	0	0	negative	29	4413	≥10
	PSM-624	0	0	negative	<10	<10	negative
	PAP-213	0	0	negative	41	25783	≥10
35	SART2-93	51	912	≥2	99	934	≥2
	Lck-488	108	61	negative	74	721	≥2
	PSA-152	0	0	negative	10	900	≥10
	PSA-248	0	0	negative	717	1058	negative
36	SART3-109	0	0	negative	184	228	negative
	Lck-208	56	0	negative	23	28	negative
	PAP-213	57	1802	≥10	13	379	≥10
	SART3-315	158	1121	≥2	NT	NT	NA
37	SART3-302	0	1417	≥10	40	11118	≥10
	SART3-309	0	0	negative	108	424	≥2
	PSA-170	0	0	negative	21	1221	≥10
	PSA-178	0	0	negative	32	1889	≥10
38	SART3-302	0	0	negative	309	15523	≥10
	CypB-129	0	0	negative	91	858	≥2
	PSMA-441	0	1163	≥10	NT	NT	NA
	PSMA-711	0	0	negative	NT	NT	NA
39	SART3-109	0	282	≥10	134	9562	≥10
	Lck-486	449	126	negative	14	12	negative
	PSA-248	157	172	negative	12	14507	≥10
	PTHrP-102	209	119	negative	16	11256	≥10
40	SART2-161	81	0	negative	1433	1451	negative
	SART3-109	0	0	negative	5368	24796	≥2
	PSA-248	0	0	negative	47	3854	≥10
	EZH2-291	0	784	≥10	2027	6674	≥2
41	SRAT3-109	0	0	negative	170	992	≥2
	Lck-488	0	0	negative	54	30278	≥10
	MRP3-1293	312	0	negative	21	3996	≥10
	PSA-248	78	0	negative	25	29669	≥10
42	CypB-129	464	0	negative	348	468	negative
	HNRL-501	0	436	≥10	859	1298	negative
	EIF-51	0	102	≥10	714	6797	≥2
	EZH2-569	0	899	≥10	2501	305	negative
43	CypB-129	140	0	negative	26	38	negative
	UBE-43	141	3424	≥10	27	1910	≥10
	EZH2-569	313	417	negative	18	446	≥10
	Her2-484	69	0	negative	<10	15	≥10

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

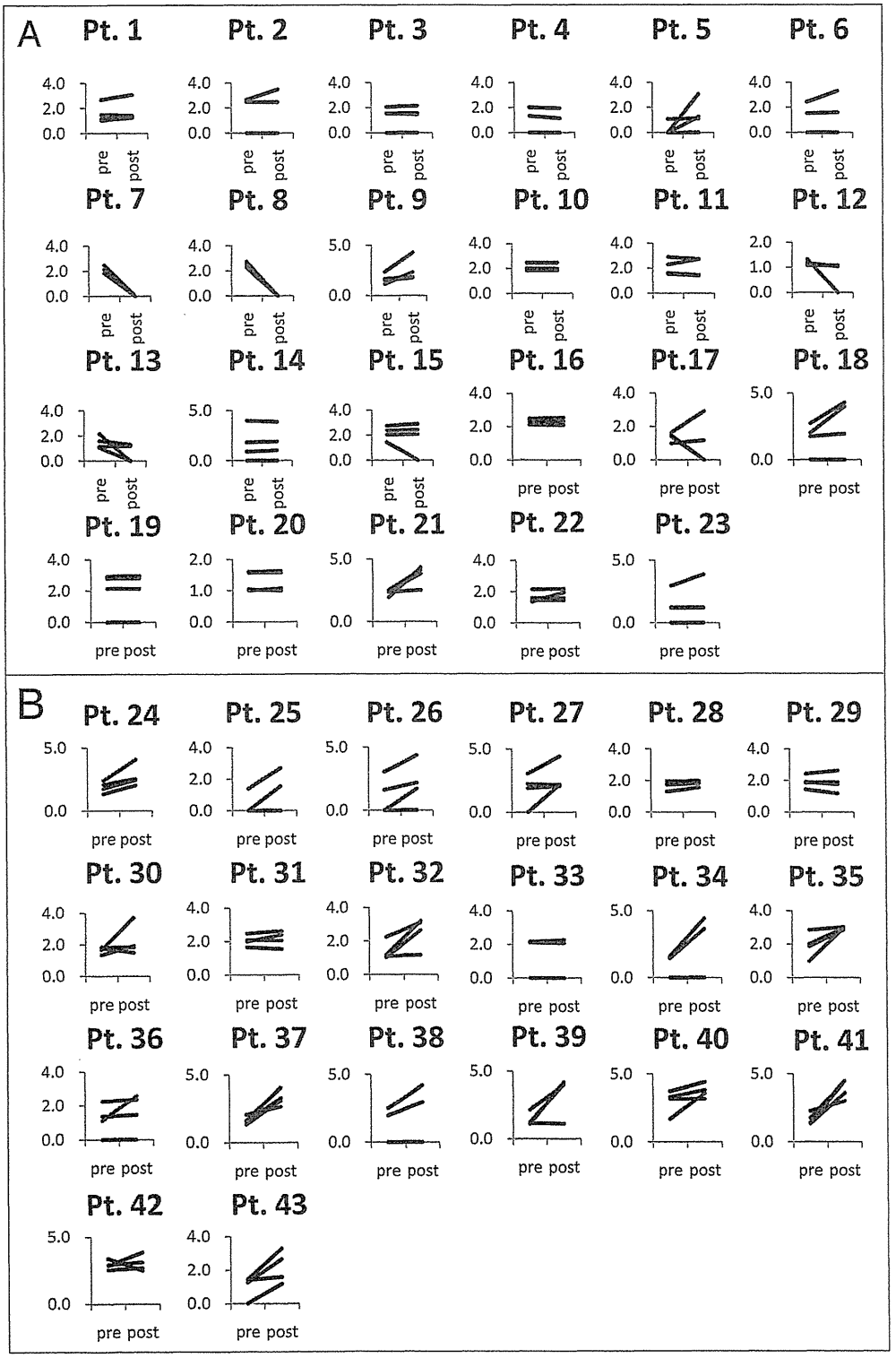

Figure 2. Changes of IgG levels reactive to each of the vaccinated peptides during pre- and post-vaccination periods (sixth) for short-term survivors (A) and long-term survivors (B). The vertical bars denote log 10 scores in order to better represent n-fold increases in IgG levels. NA, not available.

encoding tumor associated antigens by means of cDNA expression cloning technique reported by Boon et al.[40] Among many peptide candidates coded by these antigens, the peptides capable of inducing CTL reactive to tumor cells in HLA-class IA-restricted and peptide-specific manners were screened by incubation of PBMCs from cancer patients. Interestingly, many of these identified peptides were also recognized by pre-vaccination plasma IgG of cancer patients as reported previously.[41] Subsequently, to save limited source of patients' PBMCs, a large numbers of peptide candidates holding the motifs for binding to HLA-class IA molecules were at first tested for their ability to react to pre-vaccination patients' IgG, followed by testing their ability to induce HLA-class IA-restricted and peptide-specific CTL reactive to tumor cells in patients' PBMCs. Therefore, the peptides employed in this study mainly selected by their ability to be recognized by both cellular and humoral immunity. As far as we know, no other clinical trials of peptide-based cancer vaccine provided such peptides; other groups used the peptides capable of inducing only CTL without paying attention to their reactivity to IgG.

In conclusion, we have shown that IgG response is superior to CTL response as an immunological biomarker that is predictive of the overall survival of advanced cancer patients under treatment with personalized peptide vaccination. These results might provide new insights to better understand biomarkers of cancer vaccine for advanced cancer patients. Application of these results for the other types of cancer vaccine using common proteins or common peptides in a non-personalized manner could be worthy to consider.

Patients and Methods

Study population. This study was conducted through the serial collection of blood samples from 500

consecutive patients positive for HLA-A24, -A2 or -A3 supertypes with advanced cancer, who entered into phase I, I/II and II clinical trials for personalized peptide vaccination at 8 institutions (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, Hokkaido University Hospital, Niigata University Hospital, Kitasato University Hospital, Kansai Medical University Hospital and Yamaguchi University Hospital, Japan) between October 2000 and October 2008. The ethics review committee of each institution accepted the present project and blood samples were collected at baseline (before vaccination), at sixth vaccination, and during the follow-up period after written informed consent was obtained. All 500 patients suffered from advanced cancer originating in the prostate (n = 174), colon and rectum (n = 74), pancreas (n = 50), stomach (n = 42), brain (n = 33), uterus (n = 28), lung (n = 22), kidney (n = 13), skin (n = 12), breast (n = 11), bladder and urinary tracts (n = 10) and elsewhere (n = 31) (**Table 1A and B**). The safety, immune responses and clinical responses in most of those studied had been reported previously.[6,13-29] The exceptions were the results of vaccinations against bladder cancer, breast cancer, some pancreatic cancer cases, and those from HLA-A3 supertype-positive patients. These unpublished results have now been submitted for publication or are under preparation based on results obtained after October 2008. In the sub-analysis, 20 patients who survived more than 900 days (long-term survivors) and 23 patients who died within 300 days (short-term survivors) were selected to compare immune responses from a total of 174 patients with CRPC.

Personalized peptide vaccination and immunological assessment. Personalized peptide vaccination is based on a pre-vaccination measurement of peptide-specific CTL precursors and anti-peptide IgG in the circulation of cancer patients reactive to vaccine candidates, followed by administration of only reactive peptides (up to four peptides) as reported previously.[25-29] Selected peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and four peptides of 1.5 ml emulsion each at doses of 3 mg/peptide were injected subcutaneously into the regional lymph node area. A total of 77 candidate peptides (32 peptides for HLA-A24-positive cancer patients, 37 for HLA-A2 and 8 for HLA-A3 supertypes) were used in the personalized peptide vaccination. All of these peptides can induce HLA-A24-, A2- and A3-supertype-restricted and tumor-specific CTL activity in PBMCs of cancer patients.[6,13-29,42-44]

Before the first vaccination and 7 days after every sixth vaccination, 30 ml of peripheral blood was obtained and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using the previously reported culture method.[25-29] Briefly, PBMCs (1 x 10^5 cells/well) were incubated with 10 μM of a peptide in 200 μl of culture medium in u-bottom 96-well microculture plates (Nunc, Roskilde, Denmark). Half of the medium was removed and replaced with a fresh medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to produce IFNγ in response to CIR-A2402 or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYL RQQ LLG I for HLA-A24 and LLF GYP VYV for HLA-A2) as a negative control. For HLA-A3 supertype-positive cases, the cells were harvested and tested for their ability to produce IFNγ in response to CIR-A1101, -A31012 or -A3303 cells that were pre-loaded with either a corresponding peptide or an HIV peptide (RLR DLL LIV TR) as a negative control. The level of IFNγ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All assays were performed in quadruplicate. A two-tailed Student's t-test was employed for the statistical analyses.

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously reported.[25-29,45] In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE to wells and was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-PBS to each well. Fifty microliters of sample was used for detection with the Luminex™ system.

For evaluation of immune responses during the treatment, peptide-specific CTL precursors among PBMCs and serum levels of peptide-specific antibodies were measured every sixth vaccination. Positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 2 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 2. In addition, in the analysis between long- and short-term survivors, positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 10 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 10.

Adverse events and clinical responses. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical responses were evaluated on the basis of clinical observations and radiological findings. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Statistical methods. Overall survival and 1 and 3 year survival rates were determined by Kaplan-Meier actuarial analysis and the difference between survival curves was assessed by the log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify combinations of factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using the overall mean values as cut-off levels. All statistical calculations were carried out using the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

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Disclosure Statement

Although all authors completed the disclosure declaration, the following authors indicated a financial or other interest that is relevant to the subject matter under consideration in this article.

Employment or Leadership Position

Akira Yamada is a part-time executive of Green Peptide Co.; Consultant or Advisory Role: Kyogo Itoh, Green Peptide Co.; Stock Ownership: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Honoraria: none; Research Funding: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Expert Testimony: none; Other Remuneration: none.

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A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin

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Study Type – Therapy (case series)
Level of Evidence 4

What's known on the subject? and What does the study add?
This phase I study showed the safety and boosted immune responses of personalized peptide vaccination for advanced urotherial carcinoma.

This study showed feasibility of personalized peptide vaccination as a new therapeutic modality for advanced urotherial carcinoma patients who failed cisplatin-based chemotherapy.

OBJECTIVE

- To investigate the safety and immune responses of 12 consecutive weeks of once-weekly personalized peptide vaccine (PPV) administration in patients with advanced urothelial carcinoma (UC) for whom therapy with methotrexate, vinblastine, adriamycin and cisplatin (MVAC) has failed.

PATIENTS AND METHODS

- A phase I trial was designed. Ten patients with MVAC-refractory advanced or metastatic UC were treated with weekly personalized peptide vaccine 12 times using positive peptides chosen from 14 and 16 peptides in patients with human leucocyte antigens A24 and A2, respectively.
- Peptide-specific cytotoxic T lymphocyte precursor analysis by interferon-γ production and peptide-reactive immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay was monitored during the treatment.

RESULTS

- The peptide vaccination was safe and well tolerated with no major adverse effects. Increased cytotoxic T lymphocyte response and the anti-peptide IgG titre were revealed by the post-vaccination sera in eight patients.
- Clinical responses were as follows: one complete response, one partial response, two stable disease and six progressive disease.
- Median progression-free survival and overall survival were 3.0 and 8.9 months, respectively. In the four responders, median progression-free survival and overall survival were 21 and 24 months, respectively.

CONCLUSIONS

- This phase I study showed the safety of and boosted immune responses in response to PPV for advanced UC.
- The potential efficacy of 12 consecutive weekly vaccinations with PPV in patients with advanced UC merits further investigation based on these findings.

KEYWORDS

urothelial carcinoma, bladder cancer, peptide vaccine, personalized therapy, phase I clinical trial

INTRODUCTION

The currently available standard chemotherapy for advanced or metastatic urothelial carcinoma (UC) is a cisplatin-based treatment that includes methotrexate, vinblastine, adriamycin and cisplatin (MVAC) or gemcitabine and cisplatin [1–4]. However, there are no established therapeutic modalities for patients with UC who fail with these cisplatin-based therapies. Therefore, new approaches should be taken, and one of them could be specific immunotherapy. Recent advances in tumour immunology have resulted in the identification of a number of antigens and their peptides that are recognized by tumour-reactive and human leucocyte antigen (HLA) class I-restricted

cytotoxic T lymphocytes (CTL) [5]. Cancer vaccines have emerged as a promising therapeutic approach [6]. The efficacy of intravesical BCG in the treatment of superficial disease suggests a role for developing immune recognition strategies to enhance the treatment of UC. The presence of tumour-infiltrating CD8 T cells has been associated with survival in patients with UC [7]. CD8-expressing T cells can also recognize the NY-ESO-1 antigen [8], which occurs in approximately 30–40% of muscle-invasive bladder cancer. A recent clinical trial found that all six of six patients developed antigen-specific immune responses when treated with NY-ESO-1 vaccine [9]. Additional work evaluating the impact of immunomodulating therapy is ongoing, including the use of the anti-cytotoxic T-lymphocyte antigen-4 antibody to overcome inhibitory signals down-regulating T cells [10]. However, their clinical responses have been limited. To overcome this limitation, we devised a new regimen of peptide-based vaccination that consists of measuring pre-existing CTL precursors and IgG reactive to many kinds of vaccine candidates, followed by administration of the positively reactive peptides (personalized peptide vaccination: PPV) [11–14]. A recently conducted randomized clinical trial of PPV for advanced prostate cancer patients showed a favourable clinical response in the vaccinated group [15], whereas most of the other randomized cancer vaccine trials failed to obtain better clinical responses in the vaccine group [16–18]. In this phase I study, we addressed the feasibility of PPV for patients with advanced UC for whom MVAC therapy had failed.

PATIENTS AND METHODS

Eligible patients were included if they were ≥18 years of age with HLA-A24 and/or HLA-A2 status, as determined by commercially available serological tests (SRL, Tokyo, Japan), and were measurable or assessable and histologically proven to have locally advanced (≥T3, N1) or metastatic (M1) UC that included the urinary bladder and upper urinary tract. All patients received surgical treatment or biopsy and MVAC therapy had failed. Previous chemotherapy with radiation therapy for local treatment of the primary lesion was allowed if completed at least 4 weeks before enrolment. Patients were eligible if their disease had progressed at any time after therapy for advanced or metastatic disease or within 12 months of neoadjuvant or adjuvant treatment. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 1, adequate bone marrow reserve (white blood cell count ≥3000/μL, lymphocyte count ≥1200/μL, platelets ≥75 000/μL and haemoglobin ≥10 g/dL), hepatic function (serum bilirubin ≤1.5 mg/dL), and renal function (serum creatinine ≤1.5 mg/dL), and an estimated life expectancy of at least 12 weeks. Patients with non-malignant systematic disease that precluded them from receiving therapy, including active infection, autoimmune disease, any clinically significant cardiac arrhythmia, or congestive heart failure were not eligible. Patients also had to be negative for hepatitis B and C antigens. Patients with CNS metastases, second primary malignant lesions, or clinically significant pleural effusions or ascites or who had used any investigational agent 1 month before enrolment were not eligible. The study protocol was approved by the institutional ethical review boards of Kitasato University and Kurume University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before entering this clinical trial.

The study design was for a non-randomized, open-label, phase I study in patients with advanced or metastatic UC previously treated with MVAC chemotherapy. The treatment was carried out at Kitasato University Hospital and Kurume University Hospital in the outpatients clinic. All immunological analyses were carried out at the Department of Immunology, Kurume University School of Medicine. The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA, USA) under the conditions of Good Manufacturing Practice. The peptide candidates consisted of $SART2_{93-101}$, $SART2_{161-169}$, $SART3_{109-118}$, $Lck_{208-216}$, $Lck_{486-494}$, $Lck_{488-497}$, $MRP3_{503-511}$, $MRP3_{1293-1302}$, $PAP_{213-221}$, $PSA_{248-257}$, $PSMA_{624-624}$, $EZH2_{735-743}$, $EGF\text{-}R_{800-809}$ and $PTH\text{-}rP_{102-111}$ for patients with HLA-A24, and $SART3_{302-310}$, $SART3_{309-317}$, $CypB_{129-138}$, $Lck_{246-254}$, $Lck_{422-430}$, $ppMAPkkk_{294-302}$, $ppMAPkkk_{432-440}$, $WHSC2_{103-111}$, $WHSC2_{141-149}$, $UBE2V_{43-51}$, $UBE2V_{85-93}$, $HNRPL_{140-148}$, $HNRPL_{501-510}$, $EZH2_{569-577}$, $PSCA_{21-30}$ and $EGFR_{479-488}$ for patients with HLA-A2 [8,9,13]. These peptides have the ability to induce HLA-A24-restricted or HLA-A2-restricted and tumour-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of cancer patients, and are frequently expressed in various tumour cell lines [14,15,19]. The peptides were supplied in vials containing 3 mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 (Seppic, Paris, France), and then mixed in a Vortex mixer (Fisher, Alameda, CA, USA). The ISA51 is suitable for peptide vaccination because peptides solubilized in water phase are sequestered from peptidase-containing body fluid, and slow release of the peptides from the emulsion provides sustained antigenic stimulation [20]. The resulting emulsion (maximum of four peptides per vaccination) was injected subcutaneously into the femoral area, once a week for 12 weeks. This first cycle of treatment consisted of 12 consecutive weekly vaccinations. The cycle was repeated every 12 weeks for as long as the patients agreed to continue and their condition was considered appropriate for vaccination. Toxicity was evaluated in patients who received at least one vaccination, whereas both immunological and clinical evaluations were conducted in those who received more than six vaccinations. Blood samples for studies of immune responses were obtained on weeks 0, 6 and 12 during cycle 1. Supportive care could include blood transfusion and the administration of anti-emetics and analgesics, as appropriate. Further local therapy, including other chemotherapy regimens or radiation therapy, was allowed in patients with advanced disease after assessment of response to this regimen.

To measure peptide-specific CTL precursors, 30 mL peripheral blood was obtained before and after vaccination, and PBMCs were isolated by Ficoll–Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method [21]. Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in 200 μL of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL, Grand Island, NY, USA), 10% fetal calf serum, 100 U/mL interleukin-2 and 0.1 μM minimal essential medium non-essential amino acid solution (GIBCO BRL). Half of the medium was removed and replaced with a new medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to

Characteristics	No. of patients
Gender	
Male	8
Female	2
HLA typing	
A-2	4
A-24	5
A-2 and A-24	1
Primary organ	
Bladder	7
Upper urinary tract	2
Both	1
Surgical management	
TURBT	7
Nephroureterectomy	2
Radical cystectomy	1
Main target tumour	
Lymph node	5
Bladder	3
Bone	2
Previous treatment	
Chemotherapy	5
Chemotherapy and radiation therapy	5
Performance status*	
0	5
1	5

TABLE 1
Patient characteristics

HLA, human leucocyte antigen; TURBT, Transurethral resection of bladder tumour.
**Performance status by Eastern Cooperative Oncology Group score.*

produce interferon-γ (IFN-γ) in response to CIR-A2402 (kindly provided by Dr M. Takiguchi, Kumamoto University, Japan) or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYLRQQLLGI for HLA-A24 and LLFGYPVYV for HLA-A2) as a negative control. The level of IFN-γ was determined by ELISA (limit of sensitivity: 10 pg/mL). All assays were performed in quadruplicate. A two-tailed Student's *t* test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to an HIV peptide, and when the mean amount of IFN-γ production in response to a corresponding peptide was >50 ng/mL compared with that in response to an HIV peptide. The increment of CTL activity was judged as positive if the post-vaccination sample, but not the pre-vaccination sample, showed CTL activity. It was also judged as positive if the level of IFN-γ produced by the post-vaccination (12th) sample was twice as high as that produced by the pre-vaccination sample. Our previous study showed that both increased IgG and a CTL response at least twice that of the vaccinated peptides correlated well with overall survival in patients with castration-resistant prostate cancer [22].

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously reported [23]. In brief, plasma was incubated with 25 μL peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μL biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, 100 μL of streptavidin-phycoerythrin was added to the wells, and the mixture was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μL Tween-PBS into each well. Fifty microlitres of sample was detected using the Luminex™ system. The sample was judged to be positive if the IgG level of the post-vaccination (12th) plasma was twice as high as that of the pre-vaccination plasma. This definition is the same as the CTL response according to our previous results [22].

Standard indirect immunoperoxidase procedures (ENVISION Kit; DakoCytomation California, Carpinteria, CA, USA) in combination with monoclonal antibodies were used for the detection of infiltrating lymphoid cells (CD45RA and CD45RA, 1:50; Dako, Glostrup, Denmark) [24]. Cells with known positive results were used as positive controls. The primary antibody was omitted for negative controls.

Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical response was evaluated based on clinical observations and radiological findings. All known sites of disease were evaluated every 6 weeks by CT scan or MRI examination before and after each cycle. During treatment, blood counts and serum chemistries were performed weekly. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Student's *t* test was employed for evaluation of immunological assays. Progression-free survival time, overall survival time and response duration were calculated from the first day of peptide vaccination until the date of disease progression or death. The time-to-event endpoint was derived by the Kaplan–Meier method. All patients entering the trial were included in the survival determinations.

RESULTS

Between July 2007 and April 2009, 10 patients were treated with peptide vaccination at our institutions. Data were collected until December 2009. One patient did not meet the protocol entry criteria because cisplatin-based chemotherapy had not been received before the peptide vaccination. Median age was 71 years (range 44–77 years). Median follow-up time was 8.9 months (mean 12.0 months, range 2.5–29.3 months). Seven patients had bladder UC, two patients had upper urinary tract UC and one patient had bladder and upper urinary tract UC. Seven patients had metastatic disease, of whom five had lymph node metastasis and two had bone metastasis; three patients had locally advanced UC without distant metastasis after MVAC chemotherapy. The clinical characteristics of all entry patients are listed in Table 1.

For the selection of peptides for the first to 12th vaccinations (the first cycle), pre-

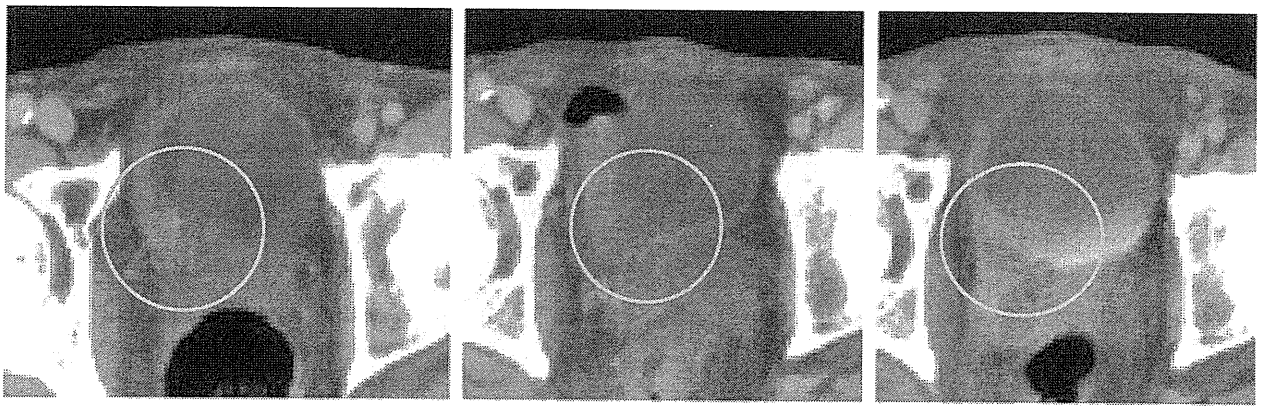
TABLE 2 Immune responses and clinical outcomes

Patient no. Clinical stage	Peptide	No. of vaccinations	Cellular response* Pre-	After 12th	Anti-peptide IgG† Pre-	After 12th	Clinical response	PFS (months)	OS (months)	Prognosis
1 T4N0M1	PAP-213	10	–	NA	1753	NA	PD	1	3	Dead
	PSA-248		–	NA	110	NA				
	EZH2-735		–	NA	51	NA				
	PTHrP-102		–	NA	149	NA				
2 T3bN0M0	SART3-109	24	–	–	193	238	PR	22	28	Alive
	Lck-486		–	–	45	43				
	MRP3-1293		–	–	128	180				
	PAP-213		–	1923	167	23 959				
3 TisN2M1	SART3-109	12	–	–	48	13 261	PD	3	5	Dead
	Lck-486		155	–	53	156				
	MRP3-1293		–	–	228	2 144				
	PAP-213		–	–	353	25 892				
4 T3bN2M0	SART3-109	25	158	137	341	26 423	SD	22	29	Alive
	Lck-488		–	327	195	769				
	PAP-213		–	207	344	22 943				
	SART2-92		68	162	214	221				
5 T3bN1M0	MAP-432	12	–	113	37	128	CR	20	20	Dead
	Lck-422		–	216	32	25				
	WHSC2-103		57	2558	15	19				
	UBE2V-85		–	2684	20	26				
6 T3bN1M0	SART3-309	12	117	198	66	61	PD	3	4	Dead
	CypB-129		–	–	99	90				
	UBE2V-43		–	–	174	303				
	HNRPL-501		–	548	55	41				
7 T4aN2M1	SART3-109	12	–	–	62	25 796	PD	3	9	Dead
	Lck-486		–	–	31	42				
	Lck-488		–	–	89	131				
	UBE2V-43		–	6212	72	272				
8 T4N2M0	Lck-422	23	–	251	47	4 315	SD	3	9	Dead
	UBE2V-43		–	–	61	12 296				
	WHSC2-141		–	–	27	44				
	HNRPL-140		–	209	30	257				
9 T3N1M0	Lck-422	12	–	–	37	1 395	PD	4	5	Dead
	UBE2V-43		–	3252	129	11 845				
	HNRPL-140		–	–	33	2 231				
10 T4N0M0	Lck-208	16	–	193	216	232	PD	3	9	Alive
	MRP3-1293		–	1712	368	438				
	PAP-213		514	551	357	3 161				
	PSA-248		–	–	711	5 588				

CR, complete response; NA, not available; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease.
**Values indicate interferon-γ (IFN-γ) production of peripheral blood mononuclear cells reactive to the corresponding peptide (pg/mL). A two-tailed Student's t test was employed for the statistical analysis. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was >50 ng/mL, compared with that to an HIV peptide. Increment of cytotoxic T lymphocyte activity was judged as positive if the post-vaccination samples, but not the pre-vaccination samples, showed the cytotoxic T lymphocyte activity. It was also judged as positive if the level of IFN-γ produced by the post-vaccination sample was more than twice as high as that produced by the pre-vaccination sample. The values showing the increment are underlined. †Plasma levels of peptide-specific IgG were measured using the Luminex™ system. Values indicated fluorescence intensity units of IgG antibodies reactive to the corresponding peptide. The sample was judged positive if the IgG level of the post-vaccination (12th) plasma was twice as high as that of the pre-vaccination plasma. The values showing positive response are underlined.*

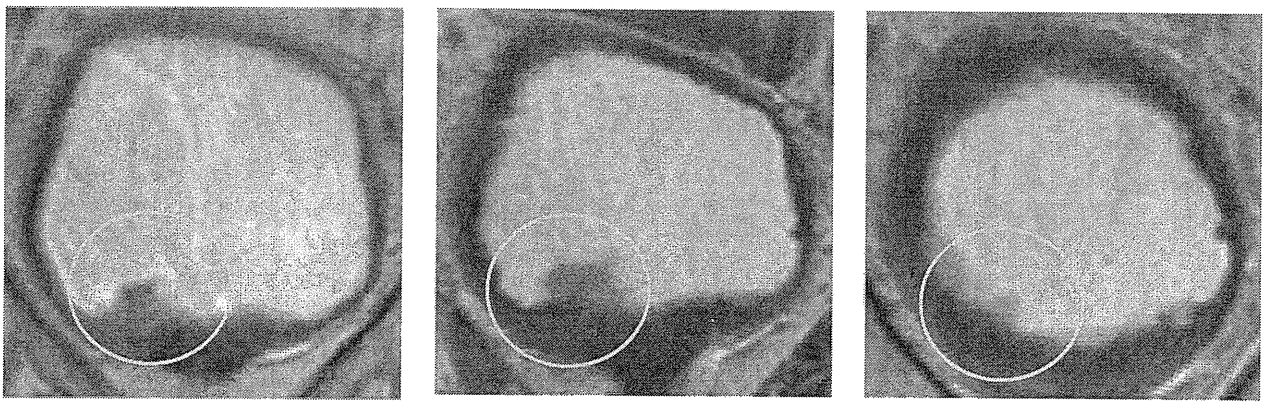
FIG. 1. The kinetic CT images of the tumour lesion of a patient with complete remission (A) and a patient with partial remission (B). The yellow circle indicates the tumour region. Left: pre-vaccination; middle: after the sixth vaccination; right: after the 12th vaccination. Cystoscopy findings of the patient with complete remission after the 12th vaccination showed no visible tumours with negative urinary cytology and post-inflammatory lesions.

(A) Complete remission



Pre-vaccination | After 6th vaccination | After 12th vaccination

(B) Partial remission



Pre-vaccination | After 6th vaccination | After 12th vaccination

vaccination plasma was used to investigate the reactivity to each of the 14 or 16 peptides in the HLA-A24$^+$ ($n = 5$) or HLA-A2$^+$ patients ($n = 4$), respectively, followed by selection of the three or four peptides with higher levels of IgG reactivity to each of the peptides in order. For the one patient who was HLA-A24$^+$ and HLA-A2$^+$, all 30 peptides were used for the selection of peptides followed by selection of three peptides from the 14 peptides used for HLA-A24$^+$ patients and the remaining one peptide from the 16 peptides used for HLA-A2$^+$ patients; the peptides chosen had the higher levels of IgG reactivity. A summary of the administered peptides is shown in Table 2. For the second cycle (13th to 24th), the four peptides with highest reactivities were similarly chosen for administration on the basis of the results of screening both PBMCs and plasma. Eight patients received twelve vaccinations and two patients received twenty-four vaccinations without other chemotherapy treatment.

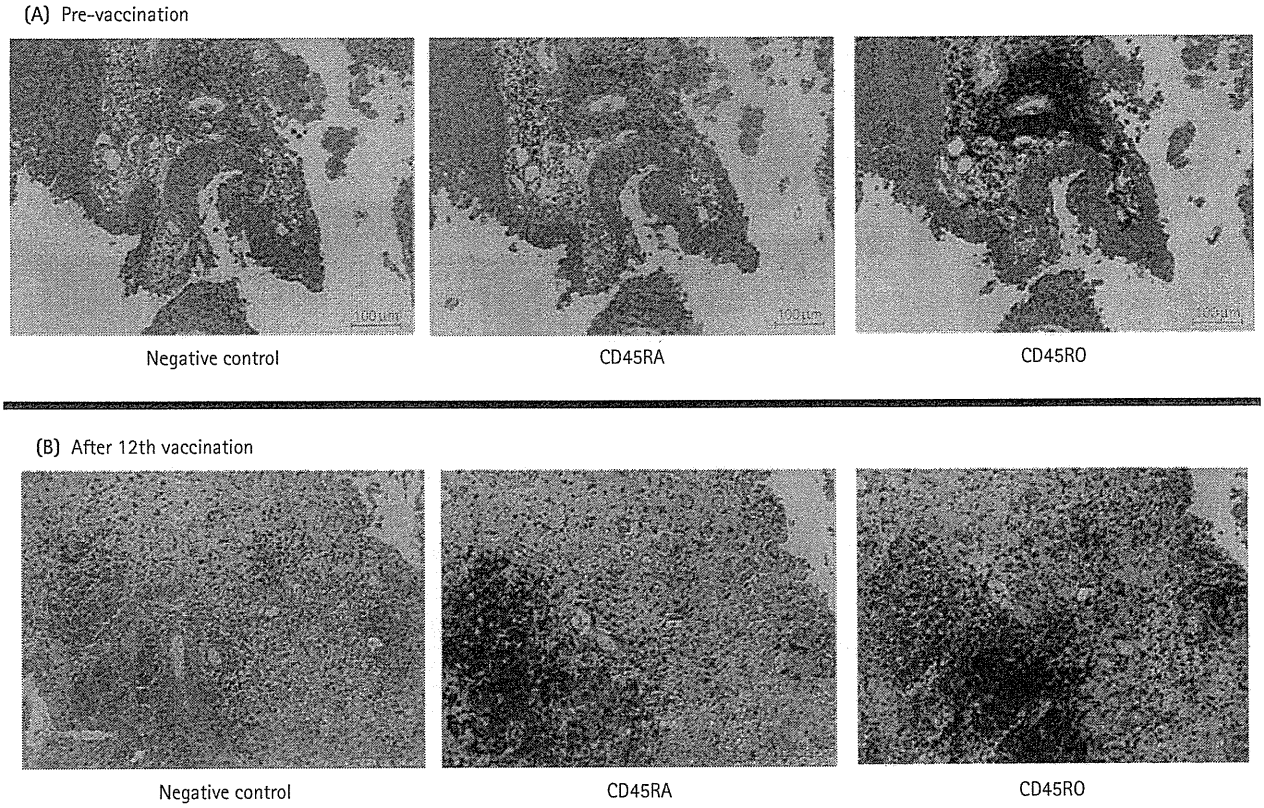
Representative non-haematological toxicity consisted of dermatological skin reactions including redness and heat at the vaccination site in all patients with grade 1 or 2 toxicity. There were no haematological toxicities or therapy-related deaths.

Peptide-specific cellular and humoral immune activities were measured at 12-week intervals for as long as the samples were available. The peptides used for vaccination and the corresponding immune responses are described in Table 2. One patient (#1) was not eligible because of rapid disease progression.

Among the nine patients tested, the augmentation of peptide-specific CTL responses in PBMCs taken after the 12th vaccination by IFN-γ production was observed in eight patients (#2, #4–10), and the augmentation of IgG responses in plasma taken after the 12th vaccination was also observed in eight patients (#2–5, #7–10). Both CTL and IgG responses were boosted in seven of nine the patients tested and CTL or IgG responses to more than two peptides were observed in four and six tested patients, respectively.

All clinical responses were confirmed by an independent review, and were as follows: one complete response, one partial response, two stable disease and six progressive disease (Table 2). A response was recorded on

FIG. 2. Representative immunohistochemical stainings of both pre-vaccination tumour regions at the first visit before methotrexate, vinblastine, adriamycin and cisplatin therapy (A) and after the 12th vaccination (B); tumour regions with anti-CD45RO and -CD45RA monoclonal antibodies are shown. The magnification was ×100.



radiological review in four patients. The remaining six patients had disease progressions. None of the six patients who had disease progression had any response to the peptide vaccinations. At the time of analyses, seven patients had died and all patients had progressed except for one patient who had a complete response but died from a cerebral infarction after complete peptide vaccination. The median progression-free survival was 3.0 months (range 0.5–14.1 months). The median overall survival was 8.9 months (range 2.5–29.3 months). Among the four responders, the median progression-free survival and overall survival were 21 (range 2.7–22.4 months) and 24 (range, 9.0–29.3 months), respectively.

It is of note that two patients (#2 and #5) with locally advanced bladder cancer showed obvious clinical responses on kinetic CT images (Fig. 1). To investigate host-tumour interaction, immunohistochemical staining of the biopsied samples taken at the first visit before MVAC therapy and after the 12th vaccination was performed. Immunohistochemical staining at the time of the first visit before MVAC therapy showed that there were a large numbers of tumour cells in the sample, whereas lymphocyte infiltration was limited in stromal lesions. $CD45RA^+$ naive lymphocytes were rare in the stromal lesions, whereas $CD45RO^+$ activated/memory lymphocytes were found around tumour vessels and stromal lesions, but not in tumour sites (Fig. 2A). Immunohistochemical staining after the 12th vaccination showed that there were very few tumour cells in the sample but many lymphoid cells with lymphoid follicles. $CD45RA^+$ naive lymphocytes were massively observed in lymphoid follicles, while $CD45RO^+$ activated/memory lymphocytes were massively observed not only in lymphoid follicles but also in the other lesions (Fig. 2B). These results suggest that PPV induced infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites, which in turn resulted in distraction of most of the tumour cells in this patient.

DISCUSSION

No severe adverse events were observed in any of the 10 patients enrolled, although all the patients developed grade 1 or 2 local dermatological reactions at the injection sites. Therefore, in terms of safety, the toxicity of the 12-week regimen of once-weekly PPVs was tolerable and acceptable for patients with MVAC-refractory UC.

With regard to peptide-induced immune reactions, an increase in peptide-specific IFN-γ production in response to at least one of the four vaccinated peptides was observed in most of the post-vaccination PBMCs (eight of nine cases), regardless of the absence ($n = 5$) or reduced levels ($n = 5$) of CTL activity in pre-vaccination PBMCs. Boosted CTL activities in response to all four peptides were seen in the

post-vaccination samples of the patient with complete remission (#5). Similarly, an increase of peptide-specific IgGs was observed in the post-vaccination plasma of most patients (eight of nine cases). There were more than 10-fold ($n = 7$) and 100-fold ($n = 6$) increases of the IgG levels in the post-vaccination samples, suggesting that clonal expansion of peptide-reactive B cells was induced by this regimen.

These results indicated that both the cellular and humoral responses were well boosted in most patients with UC under this regimen. The profile of positive peptides varied greatly from patient to patient, suggesting that the peptides suitable for use in each patient were different, which is consistent with the previously reported results in other types of cancers [11–15]. This would be because of the heterogeneous nature of the different tumours studied and the immunological diversity of the tumour-reactive CTLs in each patient.

Although cellular immunity is the predominant effector arm of antitumour responses, humoral immunity could also play an important role in host defence against cancer cells [25]. However, the mechanism of antibody production against the small vaccination peptides is unclear. One possible explanation is that pre-existing CD4 T helper type 1 cells specific to the vaccinated peptides recognize peptides loaded on HLA-class IA molecules and so facilitate both CTL induction and IgG production. Alternatively, some peptides may bind both class I and class II HLA and induce activation of CTL and T helper type 1 cells [26]. The biological roles of peptide-reactive IgGs will also need to be clarified in the near future.

This is a phase I trial designed to investigate toxicity and immune responses, but a description of the clinical responses could be important for the next stage of clinical trials. The overall response rate defined by radiological imaging is comparable to those seen in previously reported studies using chemotherapy combinations such as gemcitabine and paclitaxel [27,28]. The median survival time of our 10 patients was somewhat shorter than those reported for patients on chemotherapy regimens [27–29], but the four responders to peptide vaccination showed a median survival time of 24 months, suggesting that PPV has the potential to provide long-term survival in some patients with advanced UC.

In this study, we observed massive infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites of a PR patient after PPV, whereas they resided around vessels and connective tissues before the vaccination (at the first visit). We previously reported that PPV induced infiltration of $CD45RO^+$ lymphocytes, but neither $CD8^+$ T cells nor $CD20^+$ B cells, in tumour sites of patients with prostate cancer [24]. In considering CD45RO expression in activated or memory T cells and CD45RA expression in naive T cells [30], PPV induced infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites, which in turn resulted in destruction of most tumour cells in this patient. Further studies with other patients' samples will be needed to clarify this issue.

The potential efficacy of 12 consecutive weekly vaccinations with PPV in patients with advanced UC merits further investigation based on the safety and boosted immune responses shown herein.

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CONFLICT OF INTEREST

None declared.

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